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Man-made chemicals such as pesticides, polychlorinated biphenyls (PCBs), phthalate esters, and dioxin have been implicated in the etiology of breast cancer. Many xenobiotics such as DDT and PCBs have weak estrogenic activity and may enhance breast cancer formation by an estrogenic effect on breast epithelial cell growth. These agents also inhibit gap junctional intercellular communication (GJIC). This inhibition may contribute to the enhancement of breast epithelial growth and breast cancer formation by xenobiotics. The studies of this project have investigated the link between xenobiotic inhibition of human mammary epithelial cell GJIC, growth, and estrogenicity. These studies are highly relevant to the prevention of breast cancer. An understanding of the relationship between xenobiotic inhibition of GJIC, estrogenic activity, and the enhancement of growth in human breast epithelial cells will lead to more widely acceptable, mechanism-based arguments that xenobiotics are involved in the etiology of human breast cancer. This should lead to more focused regulatory efforts to reduce exposure to these agents.

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I. INTRODUCTION

Many environmental chemicals such as pesticides, phthalate esters, polychlorinated biphenyls, and dioxins have been implicated as etiologic factors in human breast cancer and are also weakly estrogenic (1-7). Gap junctional intercellular communication (GJIC) is an important mechanism of cellular homeostasis and growth regulation (8). Many of the xenobiotics implicated in the etiology of breast cancer also inhibit GJIC and this effect might contribute to their carcinogenic actions (9,10). No studies, however, have investigated the relationships between the inhibition of GJIC, growth, and estrogenic activitiy of xenobiotics in human mammary epithelial cells. **The purpose of this project was to determine whether DDT-related congeners block GJIC and/or enhance growth in human mammary epithelial cells and how these effects correlated with estrogen receptor (ER) status, neoplastic transformation, and estrogenicity of the agent. We hypothesized that pesticides that block GJIC will stimulate cell growth irrespective of estrogenicity, estrogen receptor status, and neoplastic transformation.**

II. BODY

A. Background:

Man-made chemicals such as pesticides, polychlorinated biphenyls (PCBs), phthalate esters, and dioxin have been implicated in the etiology of breast cancer (1-5). Several studies have identified increased levels of xenobiotics such as *p,p'*-DDE, the major human metabolite of DDT, in women with breast cancer (2-5). Many such xenobiotics have weak estrogenic activity and have been suggested to enhance breast cancer formation by an estrogenic effect on breast epithelial cell growth (6,7). However, the role of such xenoestrogens in human breast carcinogenesis is highly controversial. Several studies indicate no association between tissue levels of xenobiotics and breast cancer (11,12) and not all of the xenobiotics associated with breast cancer are estrogenic (12). Therefore, if synthetic chemicals are to be accepted as etiologic factors in breast cancer, relevant mechanisms must be demonstrated. Few mechanistic hypotheses have been tested, however.

Many of the xenobiotics that have been linked to human breast cancer (including *p,p'*-DDT and related compounds used in this project) also inhibit gap junctional intercellular communication (GJIC) (9,10). Gap junctions and expression of their protein subunits, connexins, are also frequently decreased in neoplastic cells (8). This defect may contribute to xenobiotic-induced cancer formation and the maintenance of the neoplastic phenotype.

These points also apply to the human breast. Normal human mammary epithelial cells (NHMEC) express two connexins, connexin43 (Cx43) and connexin26 (Cx26), *in vivo* (13,14). Both GJIC and Cx26 and Cx43 expression were nearly undetectable in several human breast carcinoma cell lines and primary breast carcinomas (14). Using the technique of subtractive hybridization, expression of Cx26 was identified as a potential tumor suppressor gene in human breast epithelial cells (13). When human breast carcinoma cells were transfected with Cx26 or Cx43, cell growth was decreased and the cells expressed more differentiated functions (15). In cultures of NHMEC, several pesticides including *p,p'*-DDT, decreased GJIC (16). Thus, the reduction of GJIC and Cx26/Cx43 expression has been observed in human breast carcinoma cells and NHMEC treated with xenobiotics and may contribute to carcinogenesis in the human breast.

In this project, **we hypothesized that pesticides that block GJIC will stimulate cell growth irrespective of estrogenicity, estrogen receptor status, and neoplastic status.** To test

this hypothesis, we investigated the effects of several DDT-related compounds (*p,p'*-DDT, *p,p'*-DDE, *p,p'*-DDD, and *o,p'*-DDT) on GJIC and growth of ER-positive and ER-negative normal and neoplastic human mammary epithelial cells. These agents inhibited GJIC in other types of cells (17,18) and a recent study showed that *p,p'*-DDT blocked GJIC in NHMEC, but did not examine growth effects (16). We also used ß-estradiol, an estrogenic steroid, as a positive control. We correlated changes in GJIC with cell growth, compound estrogenicity, cell estrogen-receptor status, and neoplastic transformation. We also investigated the mechanisms of action of these compounds on GJIC in HMEC. Our results have provided new information on the role of altered GJIC in breast epithelial cell growth and neoplasia and how this is related to estrogenicity.

B. Statement of Work:

The project entailed three technical objectives:

- Characterize the dose-responsive effects of DDT-related xenobiotics on GJIC in human mammary epithelial cells.
- Determine whether DDT-related xenobiotics affect the growth of human mammary epithelial cells.
- Investigate the biochemical and molecular mechanism(s) by which DDT-related xenobiotics alter GJIC in human mammary epithelial cells.

C. Experimental Methods:

- Culture of human mammary epithelial cells: Proliferating cultures of NHMEC (samples 4144 and 4678) were obtained from Clonetics, Corp. (San Diego, CA) and cultured in serum-free/phenol red-free growth medium also obtained from Clonetics. These cells were derived by Clonetics from reduction mammoplasties and consist of cells from the mammary gland terminal ducts, the most common site of breast carcinoma development. NHMEC proliferate for at least 15 population doublings *in vitro* and are cultured by routine methods. Several immortalized human mammary epithelial cell lines were obtained from Dr. Bonnie Sloane (Wayne State University). These included nontransformed MCF-10A, H-ras-transfected MCF-10AneoT, neoplastic MCF-7, and neoplastic BT-20 cells. All cell lines were cultured in phenol red-free Dulbecco's MEM/F12 medium supplemented with 5% fetal bovine serum (FBS) and gentamicin (40 µg/ml). MCF-10A and MCF-10AneoT were also cultured in the same medium with additional growth factor supplements (20 ng/ml EGF, 10 ng/ml insulin, 100 ng/ml cholera toxin, and 500 ng/ml hydrocortisone). During treatments with xenobiotics, charcoal dextran-stripped FBS was used in place of the normal FBS.
- Determination of xenobiotic toxicity in mammary epithelial cells: The toxic effects of the DDT-related compounds were determined in these cells by trypan blue dye staining. Test agents were first dissolved in dimethylsulfoxide (DMSO) then applied to the cells (1 µl/ml culture medium). Control cultures were treated with DMSO (1 µl/ml). The cultures were sampled 1, 3, and 7 d after treatment and stained with 0.4% trypan blue. Viable and nonviable (blue) cells were identified and counted microscopically as we have reported (30).
- Xenobiotic effects on mammary epithelial cell growth: Xenobiotic effects on mammary cell growth *in vitro* were determined as we have reported (19). Cells were plated into 24 well multi-well dishes (25,000 cells/well), treated with xenobiotics, and the number of cells per

well was determined on day 7 of treatment by trypsinizing the cells and counting them with a hemacytometer. Triplicate wells were sampled per xenobiotic dose.

- Dye microinjection assay for GJIC: GJIC in these cells was assayed by microinjection of fluorescent Lucifer Yellow (LY) dye as we have described (20). The cells were cultured in 35 mm dishes, treated with the test agents for 1-7 d, then microinjected. Cells were impaled with LY-filled glass micropipets and dye was loaded into the cells by iontophoresis. Cells were observed under the fluorescent microscope for evidence of dye transfer to neighboring cells. GJIC was quantified as the percentage of neighboring cells adjacent to microinjected cells that took up dye. Ten cells per dish were injected for each treatment dose and duration and triplicate dishes were run per treatment group.
- Immunostaining of Cx43 and Cx26 gap junctions: Highly specific mouse monoclonal and rabbit polyclonal antibodies to both Cx43 and Cx26 were purchased from Zymed (South San Francisco, CA) and used to stain Cx43 and Cx26 gap junctions in xenobiotic-treated mammary epithelial cells by indirect immunofluorescence as we have reported (20).
- Western blot assay of Cx43 and Cx26 protein: Cx43 and Cx26 protein levels in xenobiotic-treated mammary cells were analyzed by Western blotting as we have described (20) using the above antibodies to Cx43 and Cx26.
- Northern blot assay of Cx43 and Cx26 mRNA: Steady-state levels of Cx43 and Cx26 mRNA in xenobiotic-treated mammary epithelial cells were analyzed by Northern blotting as we have described (20). The blots were hybridized with probes generated by random primer labeling of Cx43 and Cx26 full-length cDNAs available in my laboratory. The blots were stripped and rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe to check RNA loading and transfer.

D. Results:

Technical Objective 1: Characterize the dose-responsive effects of DDT-related xenobiotics on GJIC in human mammary epithelial cells.

- Culture of human mammary epithelial cells: We successfully cultured NHMEC-4144, NHMEC-4678, and the immortalized mammary cell lines as described above. The NHMEC were passaged by trypsinization 3-4 times before they senesced; in the studies described below, we used the cells at passages 1 and 2.
- Determination of xenobiotic toxicity in mammary epithelial cells: The toxic effects of *p,p'*-DDT, *p,p'*-DDE, *p,p'*-DDD, *o,p'*-DDT, and β-estradiol at concentrations of 0.001, 0.01, 0.1, and 1 μM were determined in the above cells by trypan blue dye staining. Control cultures were treated with DMSO (final concentration of 0.1%). None of the DDT-related compounds or the estradiol were toxic (i.e., they did not increase trypan blue staining) after 1 and 7 d of treatment (data not shown).
- Dye microinjection assay for GJIC: The effects of DDT-related compounds and β-estradiol on GJIC in the mammary cells were determined after 1 and 7 d treatment and the results are shown in Figures 1-8 and Table 1. These agents either decreased or had no effect on GJIC (dye-coupling percentage). There were no apparent relationships between compound estrogenicity, ER status of the cells, growth factor supplementation, or neoplastic transformation. The nontransformed cells (NHMEC and MCF-10A cells) had higher levels of GJIC than the neoplastic cells.

Technical Objective 2: Determine whether DDT-related xenobiotics affect the growth of human mammary epithelial cells.

- Xenobiotic effects on mammary epithelial cell growth: The effects of DDT-related compounds and β-estradiol on the growth of mammary cells were determined after culturing the cells for 7 d in the presence of each agent. The results are shown in Figures 1-8 and summarized in Table 1. In the ER-negative NHMEC, MCF-10A (plus growth factors), MCF-10AneoT (plus growth factors), and BT-20 cells, the compounds did not affect cell growth. Increases in cell growth were seen, however, when MCF-10A and MCF-10AneoT cells were cultured without additional growth factors and in the ER-positive MCF-7 cells.

Technical Objective 3: Investigate the biochemical and molecular mechanism(s) by which DDT-related xenobiotics alter GJIC in human mammary epithelial cells.

- Immunostaining of Cx43 and Cx26 gap junctions; Western blot assay of Cx43 and Cx26 protein; and Northern blot assay of Cx43 and Cx26 mRNA: Cx43 mRNA and protein were readily detected in NHMEC and MCF-10A cells and were barely detectable or undetectable in BT-20 and MCF-7 cells (Figs. 9 and 10). Cx26 was not detected in any of these cell types. The effects of DDT-related compounds and β-estradiol on Cx43 expression in the mammary cells was determined after 1 and 7 d treatment and the results are shown in Table 1. Essentially, the test agents did not alter Cx43 expression in these cells. Representative Northern and Western blots are shown in Figure 10 for NHMEC (isolation 4678). Cx43-containing gap junctions were detected in these cells by immunohistochemistry. DDT-related agents had no apparent effects on gap junction localization or density (data not shown).

III. KEY RESEARCH ACCOMPLISHMENTS

- The effects of *p,p'*-DDT, *p,p'*-DDE, *p,p'*-DDD, *o,p'*-DDT, and β-estradiol on GJIC and growth of NHMEC, MCF-10A, MCF-10AneoT, MCF-7, and BT-20 cells were determined over several logs of dose.
- All of the pesticides decreased or had no effect on GJIC (Table 1). β-Estradiol also decreased GJIC in MCF-10A and MCF-10AneoT cells.
- There were no apparent relationships between this inhibition of GJIC, the estrogenic activity of the test agents, and the estrogen receptor-status of the cells.
- All of the test agents increased the growth of MCF-7 cells. *p,p'*-DDT, *p,p'*-DDD, and *o,p'*-DDD also stimulated the growth of MCF-10A and MCF-10AneoT cells, but only when they were cultured in the absence of growth factor supplements. None of the test agents affected the growth of NHMEC and BT-20 cells.
- The inhibition of GJIC in these cells did not appear to be due to decreased expression of Cx43 or Cx26 or to altered localization of gap junctions.

IV. REPORTABLE OUTCOMES

- A manuscript pertaining to these findings is being prepared for submission to *Carcinogenesis*.
- These studies were conducted in part by Kristy Warner, a Ph.D. graduate student who will graduate this year.

CONCLUSIONS:

- DDT-related pesticides enhanced cell growth in MCF-7 (ER-positive), MCF-10A (ER-negative), and MCF-10AneoT cells (ER-negative), but not in NHMEC (ER-negative) and BT-20 (ER-negative) cells. The growth enhancing effects in MCF-10A and MCF-10AneoT cells occurred only when the cells were cultured in the absence of additional growth factors.
- GJIC was observed in all cell types, but was greatest in the nontransformed NHMEC and MCF-10A cells. The DDT congeners decreased GJIC and this was not related to their reported estrogenic activity or to their effects on cell growth. β-Estradiol did not affect GJIC in these cells. The agents also did not alter connexin expression or localization.
- The above results suggest that the enhancement of normal and neoplastic human mammary epithelial cell growth by DDT-related agents occurs by ER-dependent and ER-independent mechanisms, depending upon cell type and media components, and does not involve the downregulation of GJIC.

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- Kristy A. Warner, B.S.
- Martha J. Fernstrom, B.S.
- Randall J. Ruch, Ph.D.

Table 1. Summary of the effects of DDT-related compounds and β-estradiol on growth, GJIC, connexin expression, and gap junction immunostaining in normal, immortalized, and neoplastic human mammary epithelial cells.

Cell type	ER status	Compound	Growth	GJIC at 1 d	GJIC at 7 d	Cx26 expression	Cx26 immuno.	Cx43 expression	Cx43 immuno.
NHMEC (#4144)	negative	p,p'-DDT	–	↓	–	∅	∅	–	–
		p,p'-DDE	–	–	–	∅	∅	–	–
		p,p'-DDD	–	↓	–	∅	∅	–	–
		o,p'-DDD	–	↓	–	∅	∅	–	–
		β-estradiol	–	–	–	∅	∅	–	–
NHMEC (#4678)	negative	p,p'-DDT	–	↓	↓	∅	∅	–	–
		p,p'-DDE	–	–	–	∅	∅	–	–
		p,p'-DDD	–	–	↓	∅	∅	–	–
		o,p'-DDD	–	↓	↓	∅	∅	–	–
		β-estradiol	–	–	↓	∅	∅	–	–
MCF-10A (minus GF)	negative	p,p'-DDT	↑	↓	↓	∅	∅	–	–
		p,p'-DDE	–	↓	↓	∅	∅	–	–
		p,p'-DDD	↑	↓	↓	∅	∅	–	–
		o,p'-DDD	↑	↓	↓	∅	∅	–	–
		β-estradiol	–	–	–	∅	∅	–	–
MCF-10A (plus GF)	negative	p,p'-DDT	–	↓	↓	∅	∅	–	–
		p,p'-DDE	–	↓	↓	∅	∅	–	–
		p,p'-DDD	–	↓	–	∅	∅	–	–
		o,p'-DDD	–	↓	–	∅	∅	–	–
		β-estradiol	–	↓	↓	∅	∅	–	–

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Table 1, continued.

Cell type	ER status	Compound	Growth	GJIC at 1 d	GJIC at 7 d	Cx26 expression	Cx26 immuno.	Cx43 expression	Cx43 immuno.
MCF10A-neoT (minus GF)	negative	p,p'-DDT	↑	–	–	∅	∅	–	–
		p,p'-DDE	–	–	–	∅	∅	–	–
		p,p'-DDD	↑	–	–	∅	∅	–	–
		o,p'-DDD	↑	–	–	∅	∅	–	–
		β-estradiol	–	–	–	∅	∅	–	–
MCF10A-neoT (plus GF)	negative	p,p'-DDT	–	↓	↓	∅	∅	–	–
		p,p'-DDE	–	↓	–	∅	∅	–	–
		p,p'-DDD	–	↓	–	∅	∅	–	–
		o,p'-DDD	–	↓	–	∅	∅	–	–
		β-estradiol	–	↓	–	∅	∅	–	–
MCF-7	positive	p,p'-DDT	↑	↓	↓	∅	∅	–	–
		p,p'-DDE	↑	↓	↓	∅	∅	–	–
		p,p'-DDD	↑	–	↓	∅	∅	–	–
		o,p'-DDD	↑	↓	–	∅	∅	–	–
		β-estradiol	↑	–	–	∅	∅	–	–
BT-20	negative	p,p'-DDT	–	–	↓	∅	∅	–	–
		p,p'-DDE	–	–	–	∅	∅	–	–
		p,p'-DDD	–	–	–	∅	∅	–	–
		o,p'-DDD	–	–	–	∅	∅	–	–
		β-estradiol	–	–	–	∅	∅	–	–

↑, increased.
↓, decreased.
∅, not detected.
–, no effect.

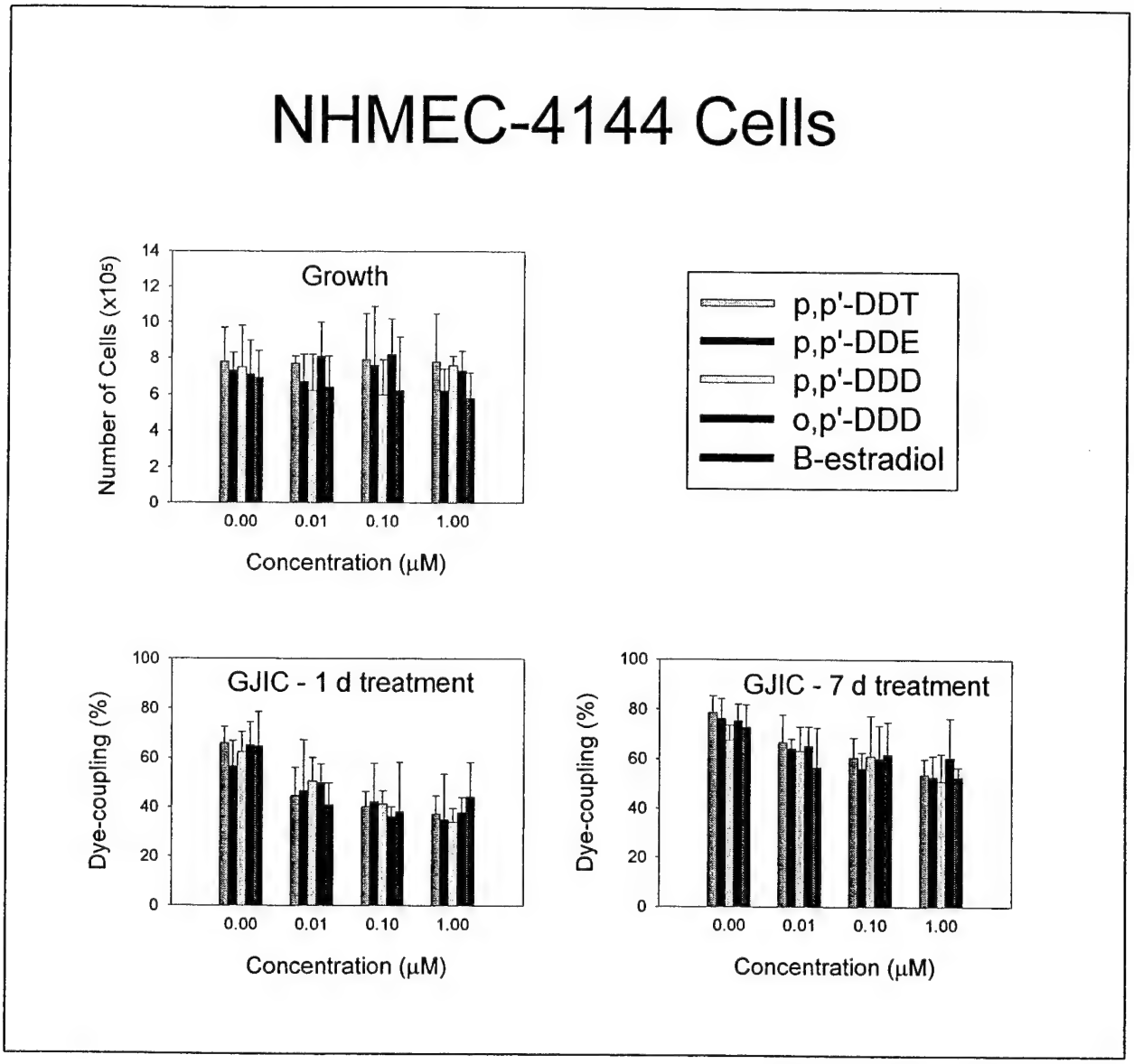

Figure 1. Effects of DDT-related compounds and β-estradiol on growth and GJIC in NHMEC (preparation #4144). Growth was measured as the number of cells per 35 mm culture dish after 7 d treatment with the test agents (mean±S.D.). GJIC was quantified as the percentage of first order neighboring cells that exhibited dye-coupling with microinjected, dye-filled cells (mean±S.D.).

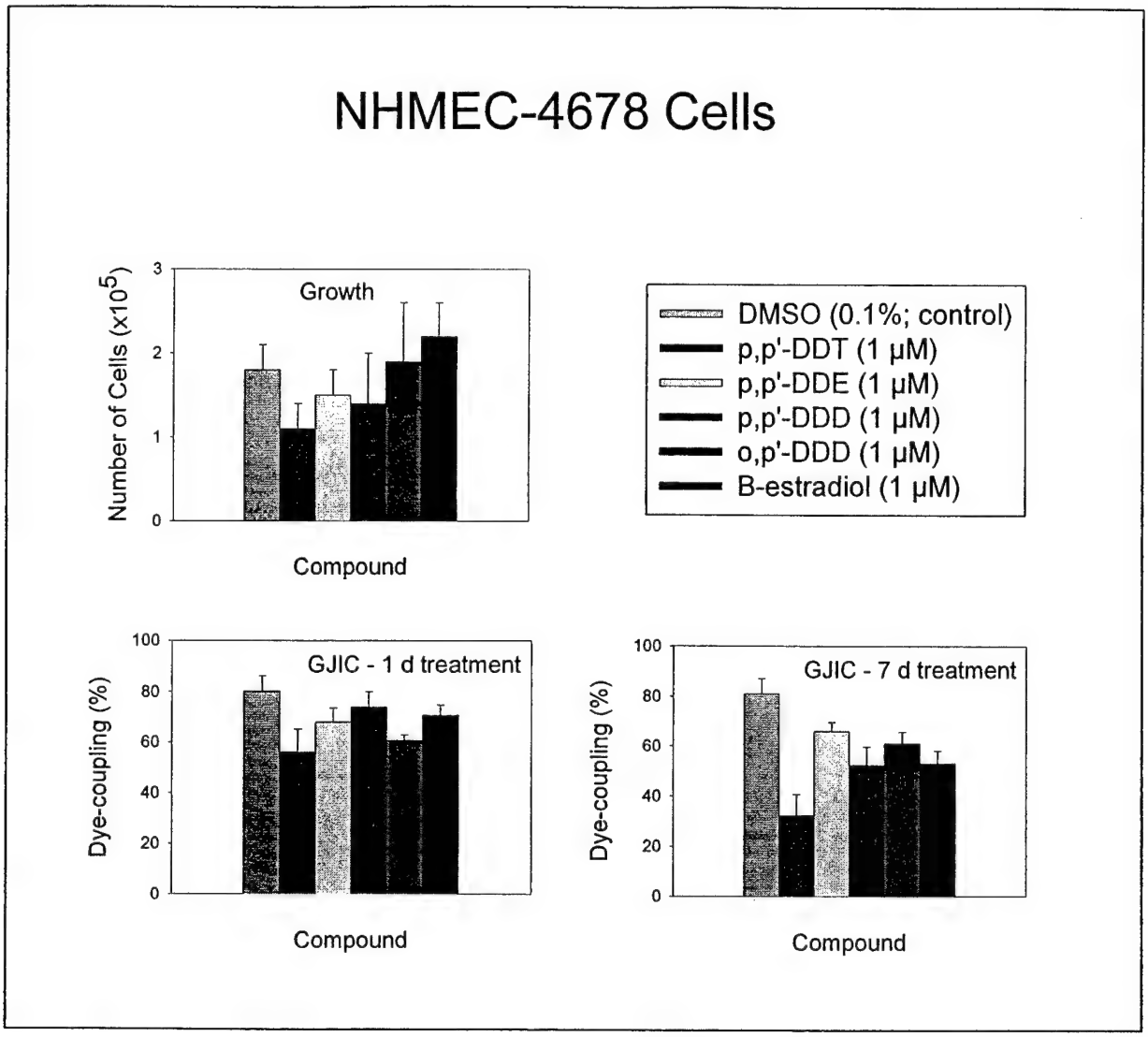

Figure 2. Effects of DDT-related compounds and β-estradiol on growth and GJIC in NHMEC (preparation #4678). Growth was measured as the number of cells per 35 mm culture dish after 7 d treatment with the test agents (mean±S.D.). GJIC was quantified as the percentage of first order neighboring cells that exhibited dye-coupling with microinjected, dye-filled cells (mean±S.D.).

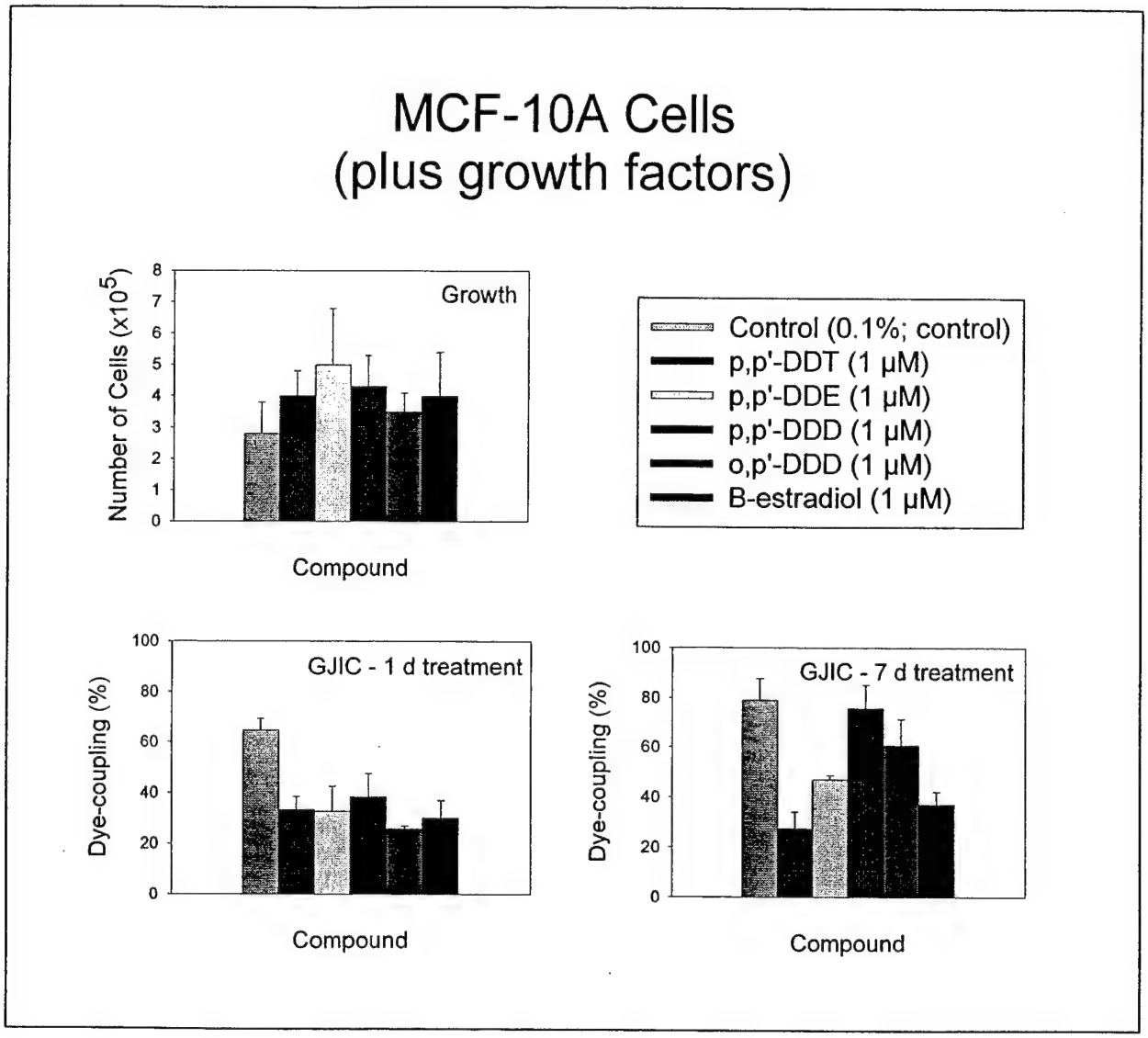

Figure 3. Effects of DDT-related compounds and β-estradiol on growth and GJIC in MCF-10A cells cultured with supplemental growth factors. Growth was measured as the number of cells per 35 mm culture dish after 7 d treatment with the test agents (mean±S.D.). GJIC was quantified as the percentage of first order neighboring cells that exhibited dye-coupling with microinjected, dye-filled cells (mean±S.D.).

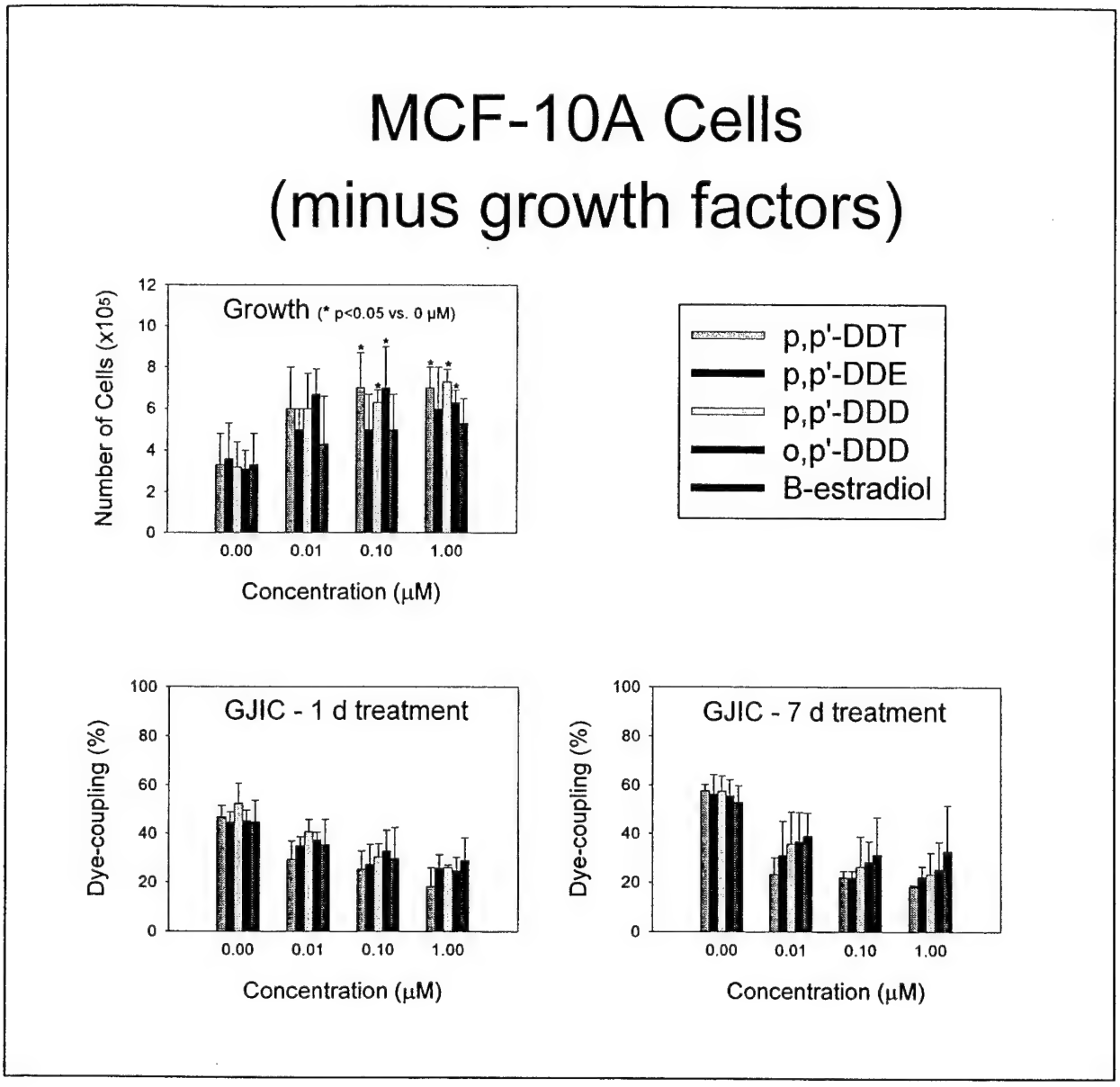

Figure 4. Effects of DDT-related compounds and β-estradiol on growth and GJIC in MCF-10A cells cultured without supplemental growth factors. Growth was measured as the number of cells per 35 mm culture dish after 7 d treatment with the test agents (mean±S.D.). GJIC was quantified as the percentage of first order neighboring cells that exhibited dye-coupling with microinjected, dye-filled cells (mean±S.D.).

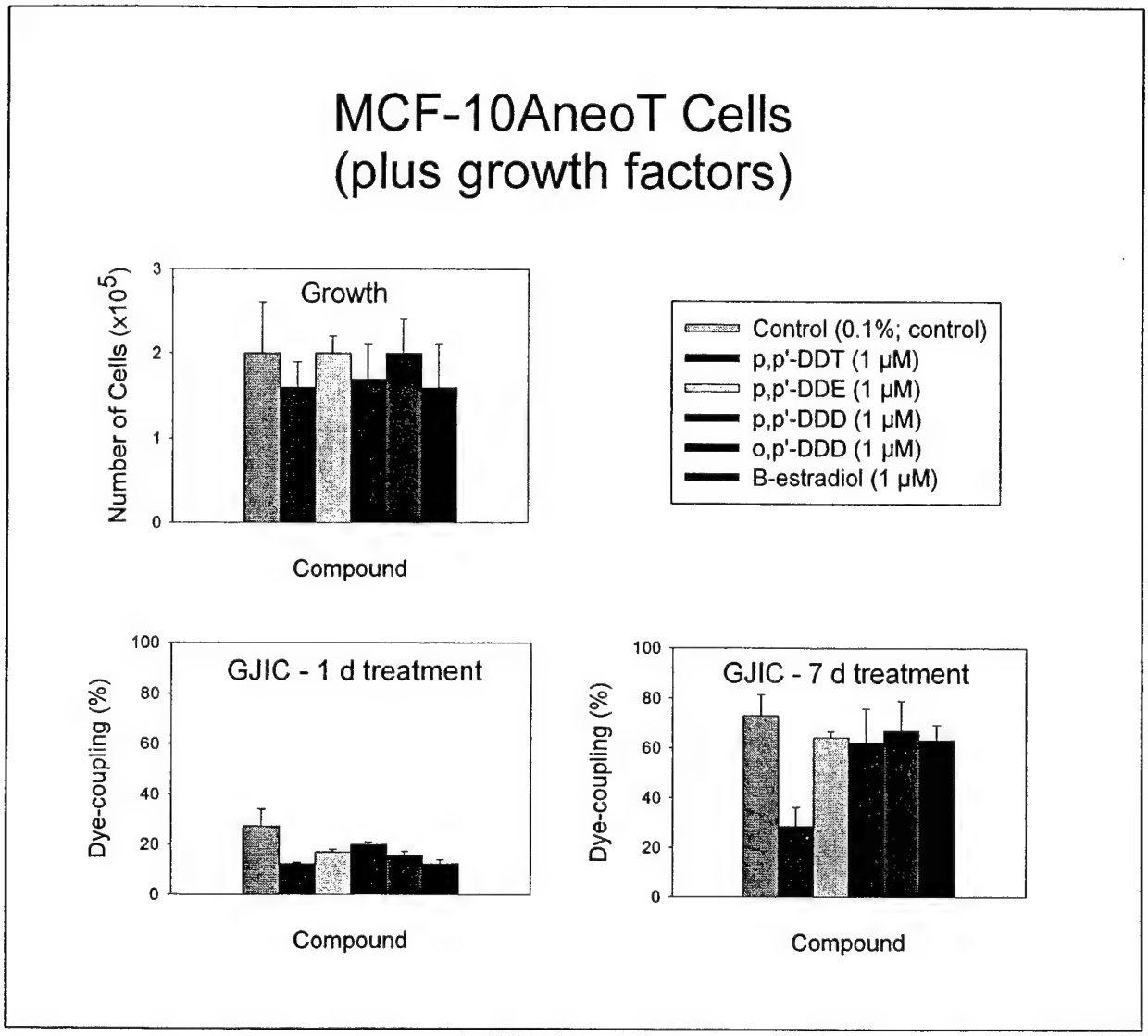

Figure 5. Effects of DDT-related compounds and β-estradiol on growth and GJIC MCF-10AneoT cells cultured with supplemental growth factors. Growth was measured as the number of cells per 35 mm culture dish after 7 d treatment with the test agents (mean±S.D.). GJIC was quantified as the percentage of first order neighboring cells that exhibited dye-coupling with microinjected, dye-filled cells (mean±S.D.).

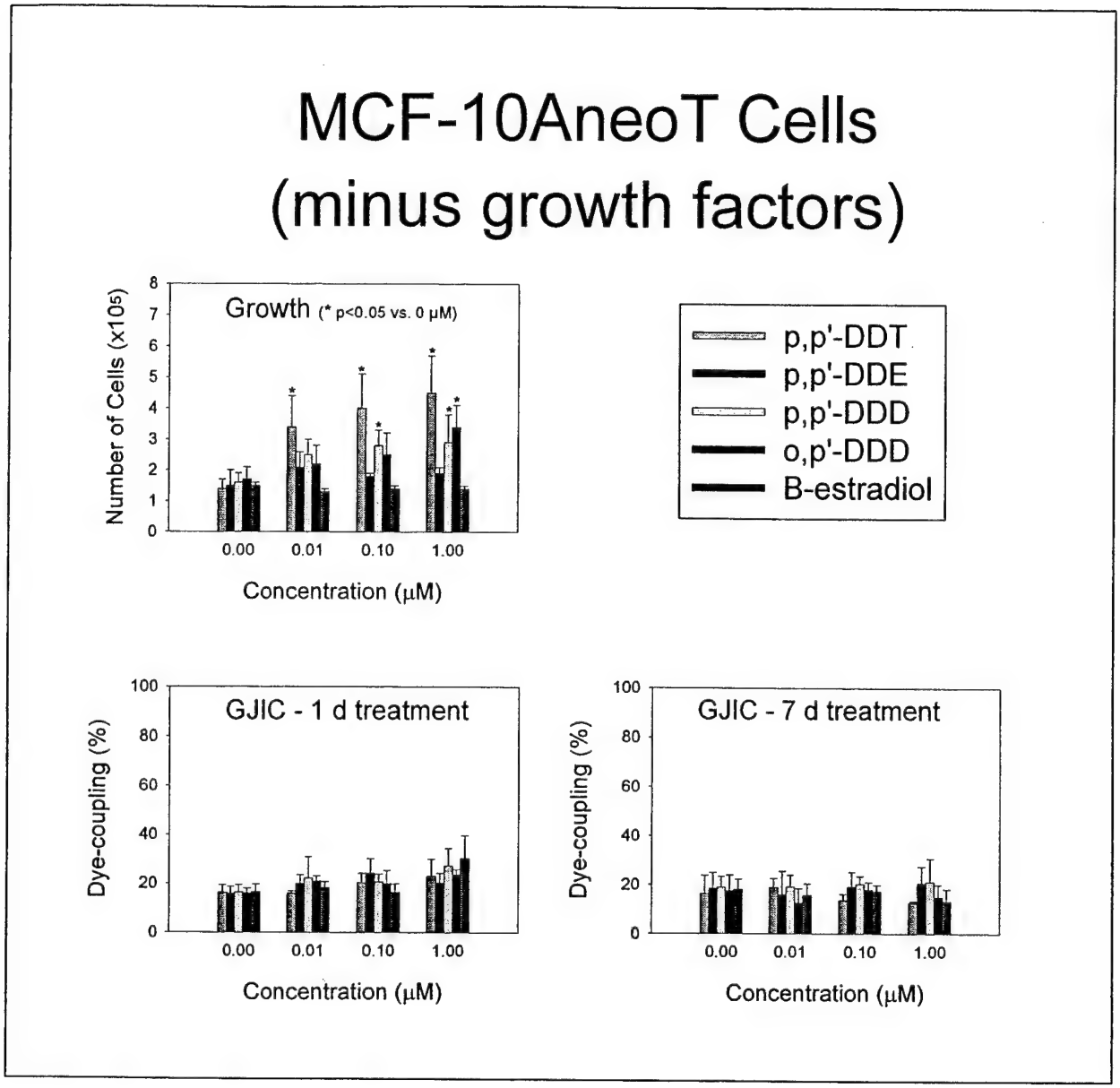

Figure 6. Effects of DDT-related compounds and β-estradiol on growth and GJIC in MCF-10AneoT cells cultured without supplemental growth factors. Growth was measured as the number of cells per 35 mm culture dish after 7 d treatment with the test agents (mean±S.D.). GJIC was quantified as the percentage of first order neighboring cells that exhibited dye-coupling with microinjected, dye-filled cells (mean±S.D.).

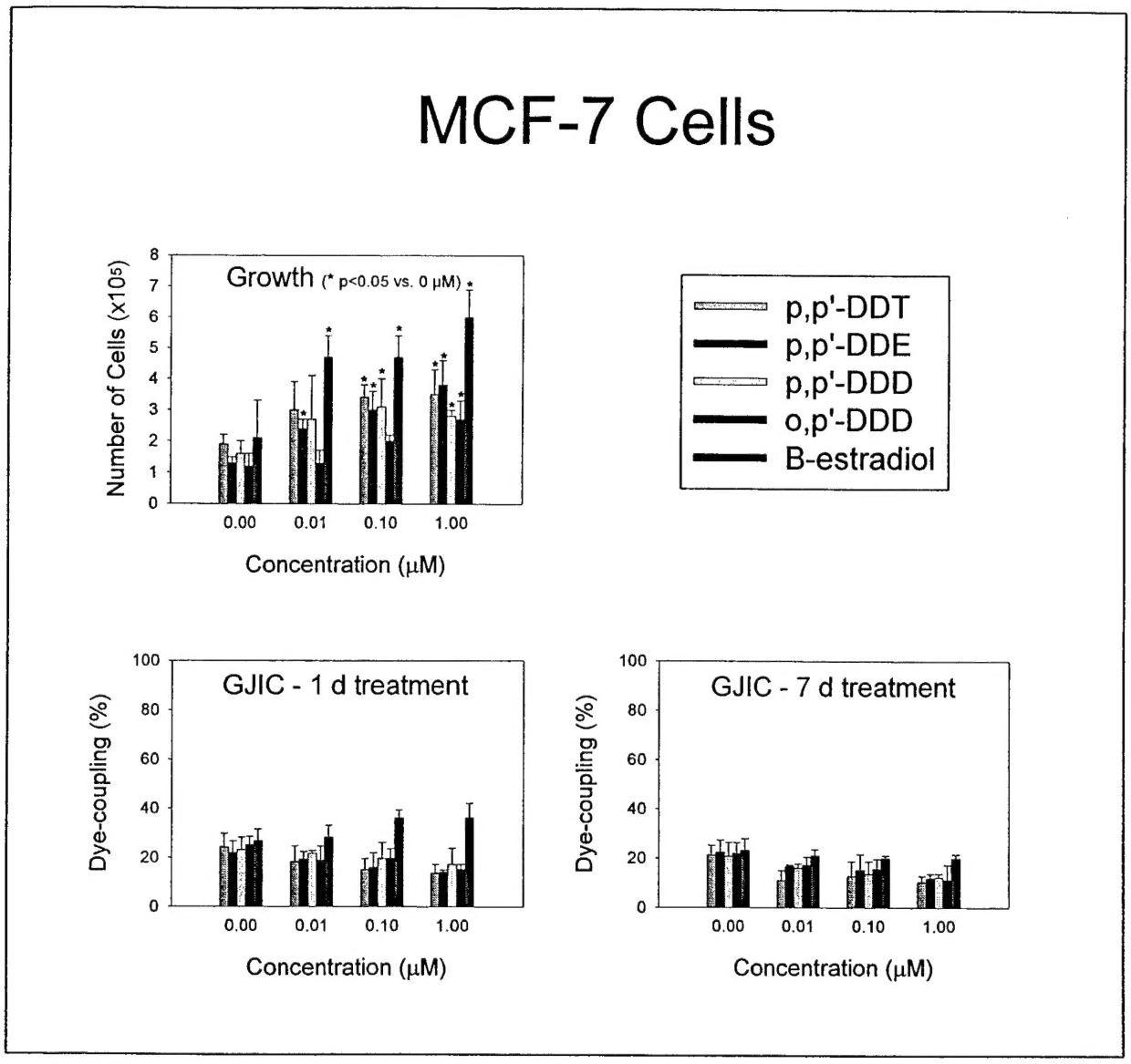

Figure 7. Effects of DDT-related compounds and β-estradiol on growth and GJIC in MCF-7 cells. Growth was measured as the number of cells per 35 mm culture dish after 7 d treatment with the test agents (mean±S.D.). GJIC was quantified as the percentage of first order neighboring cells that exhibited dye-coupling with microinjected, dye-filled cells (mean±S.D.).

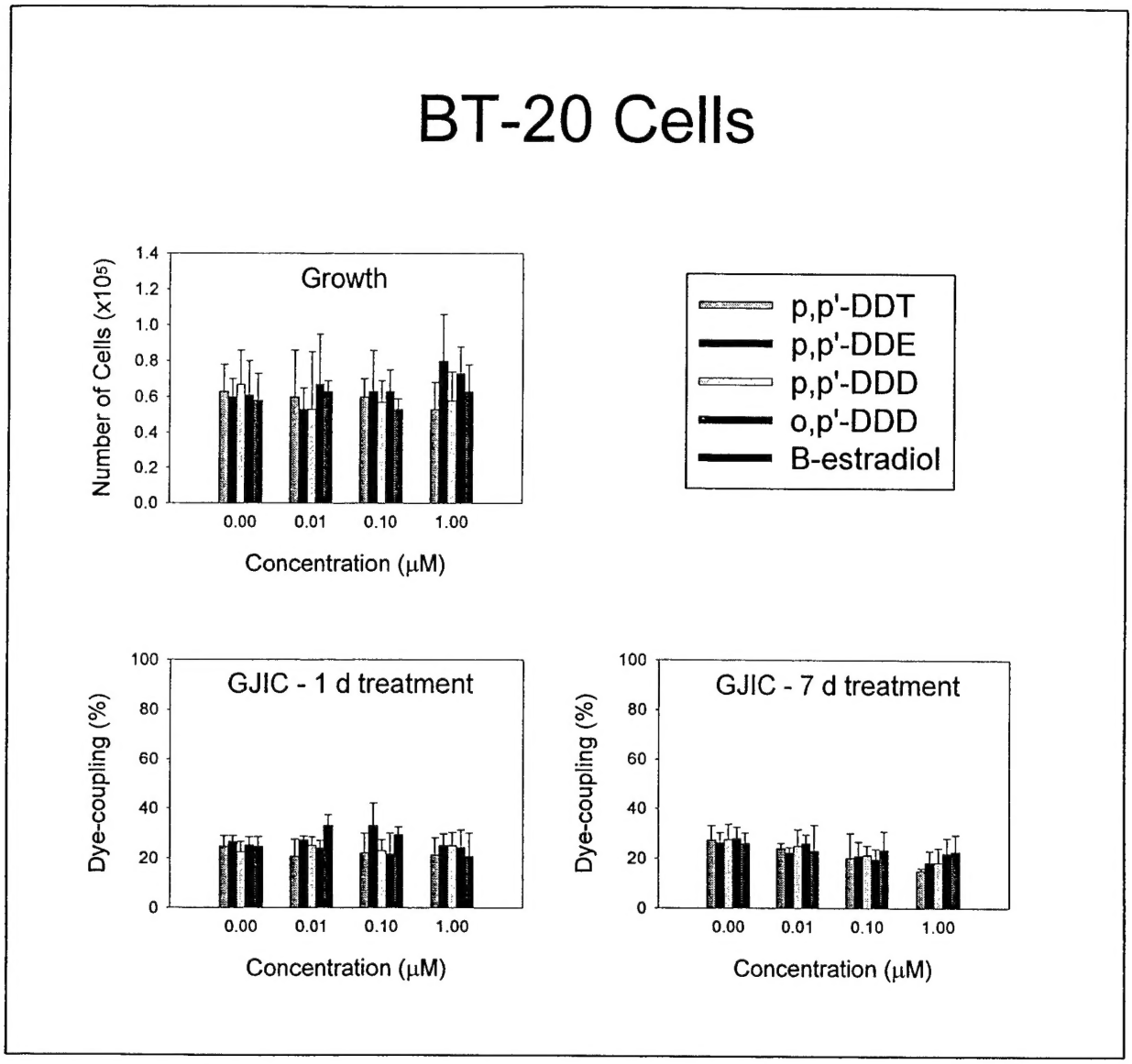

Figure 8. Effects of DDT-related compounds and β-estradiol on growth and GJIC in BT-20 cells. Growth was measured as the number of cells per 35 mm culture dish after 7 d treatment with the test agents (mean±S.D.). GJIC was quantified as the percentage of first order neighboring cells that exhibited dye-coupling with microinjected, dye-filled cells (mean±S.D.).

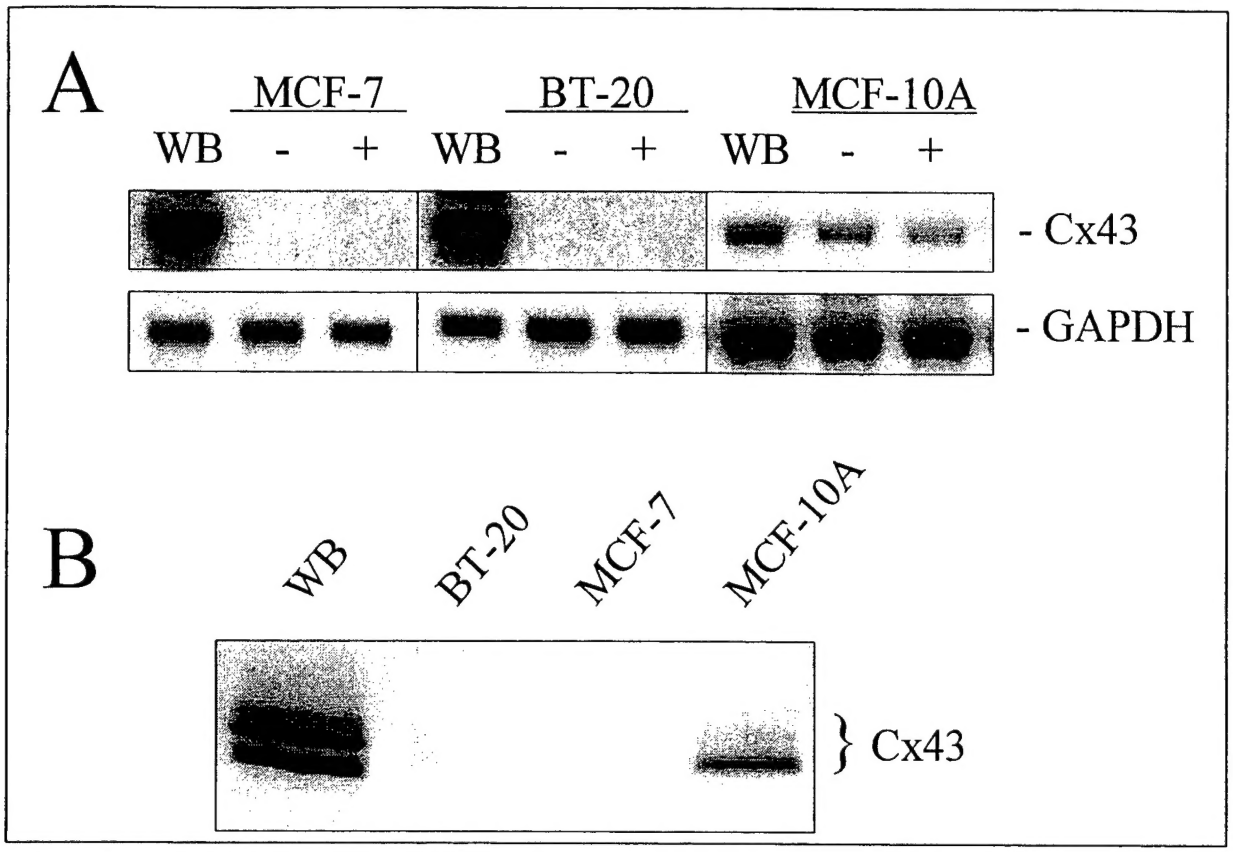

Figure 9. (A) Northern blot of Cx43 mRNA in MCF-7, BT-20, and MCF-10A cells after treatment with β-estradiol (1 μM; +) or no treatment (control; -) for 7 d. WB-F344 rat liver epithelial cells (WB) were also run as a positive control. GAPDH mRNA was detected to evaluate RNA loading and transfer. (B) Western blot of Cx43 protein in WB-F344 (positive control), BT-20, MCF-7, and MCF-10A cells. The Cx43 protein exists in several phosphorylated states and migrates on SDS-PAGE at ~42-46 kDa. The lowest band is not phosphorylated (20).

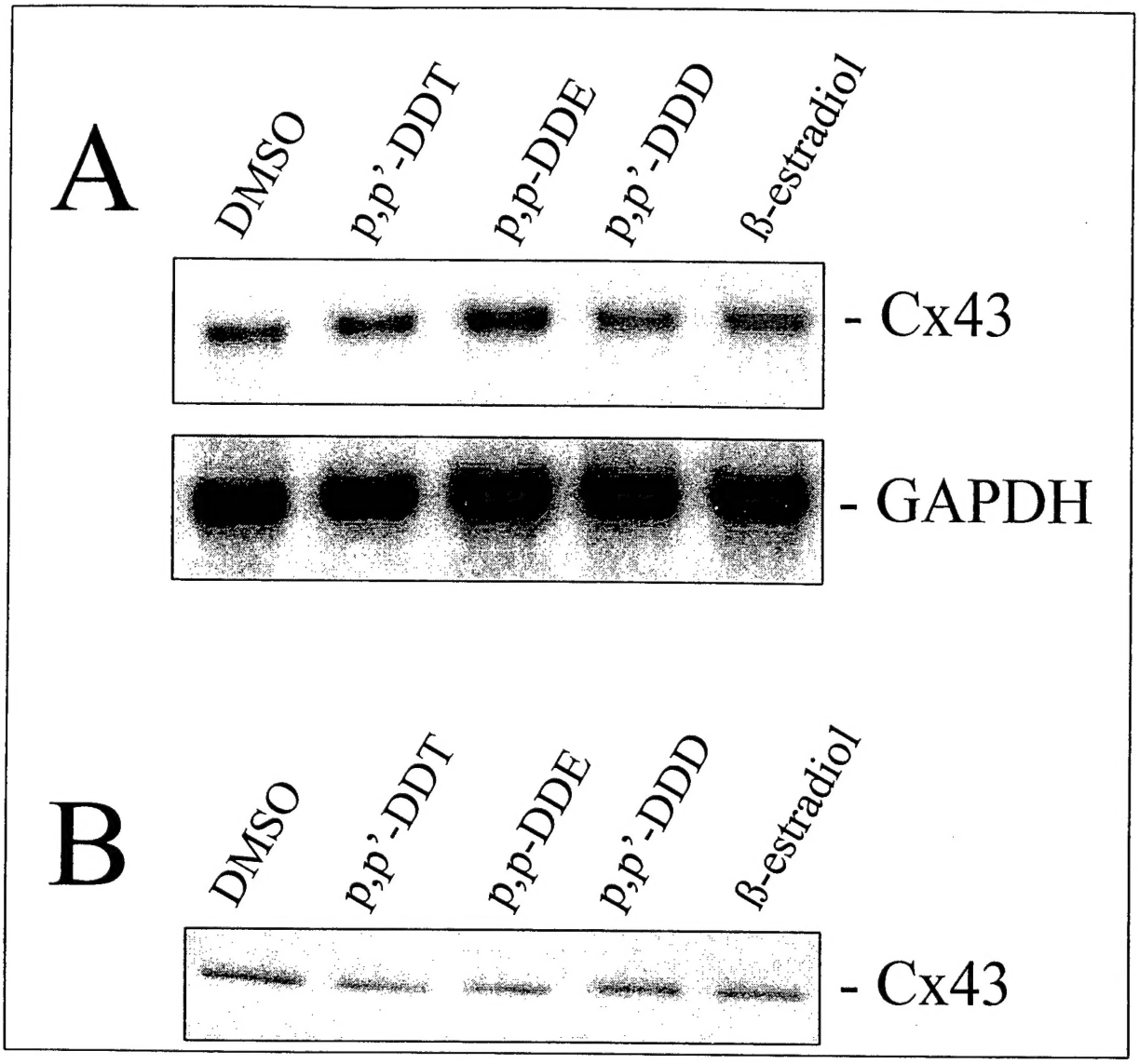

Figure 10. Northern blot of Cx43 mRNA (A) and Western blot of Cx43 protein (B) in NHMEC (preparation #4678) after treatment with DDT-related agents and β-estradiol (all 1 μM) or DMSO (solvent control; 0.1%) for 7 d. GAPDH mRNA was detected to evaluate RNA loading and transfer. The Cx43 protein migrated as a single band at ~42 kDa.